

## Delivery of Neurotrophin-3 to the Cochlea using Alginate Beads

\*Farnoosh Noushi, †Rachael T. Richardson, †Jennifer Hardman, \*†Graeme Clark,  
and \*†Stephen O'Leary

\*University of Melbourne, Department of Otolaryngology, East Melbourne, Australia, and †Bionic Ear  
Institute, East Melbourne, Australia.

**Objective:** The aim of this study was to design a novel cochlear neurotrophin (NT) delivery system for the rescue of auditory neurons after ototoxicity-induced deafening.

**Background:** NT-3 is a trophic growth factor that promotes the survival of the auditory nerve and may have a potential therapeutic role in slowing neuron loss in progressive deafness, especially as an adjunct to the current cochlear implant. Beads made from alginate are biodegradable, slow release substances that can be placed at the round window or inside the cochlea. This study investigated the loading properties, release kinetics, and implantation potential of alginate beads loaded with NT-3.

**Methods:** Alginate beads were prepared using an ionic gelation technique and postloaded with NT-3. Release of NT-3 was measured using enzyme-linked immunosorbent assay over 5 days. Alginate beads were implanted into deafened guinea pigs for 28 days, after which survival of auditory neurons was assessed.

**Results:** Enzyme-linked immunosorbent assay studies demonstrated a 98% to 99% loading of NT-3 with a slow, partial release over 5 days in Ringer's solution. Furthermore, the addition of heparin to the delivery system modulated NT-3 release to a steadier pattern. Implantation of alginate-heparin beads in guinea pig cochleae produced minimal local tissue reaction. NT-3 loaded beads implanted at both the round window and within the scala tympani of the basal turn provided auditory neurons significant protection from degradation and apoptosis compared with unloaded beads or untreated cochleae.

**Conclusions:** This study demonstrates alginate beads to be a safe, biodegradable and effective delivery system for NT-3 to the cochlea. **Key Words:** Alginate—Cochlea—Drug delivery—Neurotrophin-3—Sensorineural hearing loss.  
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Localized drug delivery to the cochlea is a promising method for the delivery of therapeutic agents that may otherwise have toxic side effects if administered systemically. One method of local delivery is diffusion of drugs through the semipermeable round window membrane. The round window is permeable to various medications, which include gentamicin for the treatment of Menière's disease (1-4) and prednisolone for idiopathic sensorineural hearing loss (5-7). The potential benefits of round window delivery of drugs include a low systemic absorption and toxicity and the ease of delivery in the outpatient setting.

Techniques developed for round window drug delivery have included blind transtympanic injections (1,2), gelfoam (8), elvax-impregnated discs (9), transtympanic

microcatheters (3,4), and the microwick made of polyvinyl (10). However, many of the methods investigated so far have limitations. Transtympanic delivery is limited by poor targeting and uncontrolled dose delivery. With the exception of gelfoam, most of the delivery systems are not biodegradable. Pump systems are limited by their cost and the requirement for hospitalization. Direct intracochlear delivery of drugs by way of pumps are an established experimental tool (11-15), but these systems are not suitable for human use because of the risk of introducing infection into the inner ear and the potential sequelae of meningitis.

We report a novel system for the delivery of drugs to the inner ear using a biodegradable and relatively inert agent, alginate-heparin beads. This system facilitates controlled and sustained drug delivery. Alginate as a delivery vehicle has been successfully used to encapsulate and deliver diverse bioactive growth factors (16) such as basic fibroblast growth factor (17,18), leukemia inhibitory factor (19), nerve growth factor (20), and ciliary neurotrophic factor (21). Furthermore, alginate has also been demonstrated to be an adequate nerve and

Address correspondence and reprint requests to Stephen O'Leary, University of Melbourne, Department of Otolaryngology, 32 Gisborne St, East Melbourne, VIC 3002, Australia; E-mail: sjoleary@unimelb.edu.au

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cellular growth matrix (22–24). Heparin was also investigated as a suitable additive to our delivery system as a modulator for release of growth factors. Heparin has been used to modulate release of growth factors such as basic fibroblast growth factor, nerve growth factor, neurotrophin (NT)-3, and brain derived neurotrophic factor from fabricated matrices in a slow and controlled manner (17,18,25–27).

In this study, we investigated the efficacy of NT-3 release from alginate-heparin beads implanted on to the round window of guinea-pig cochleae. Auditory neurons degenerate and significant numbers undergo apoptosis after any cochlear pathology, resulting in the loss of hair cells, which includes most types of sensorineural hearing loss and ototoxic injuries. NTs have been shown to promote the survival of the auditory nerve *in vitro* (28–35) and *in vivo* (13,36,37). Because the optimal functioning of cochlear implants requires the preservation of this population of auditory neurons, there is interest in the potential therapeutic use of NTs, such as NT-3, as an adjunct to implantation. For this reason, we investigated the round window delivery of NTs because this could potentially preserve the auditory neural population after deafness has occurred but before cochlear implantation.

Another potential therapeutic role for NT-3 is the prevention of ongoing apoptosis of auditory neurons after cochlear implantation. This could be achieved by the slow intracochlear release of NT-3 from polymers coating the cochlear electrode. These electrodes are implanted into the basal turn of the cochlea (in a fluid-filled chamber known as the scala tympani). To determine whether this may be feasible, we also investigated whether alginate beads introduced into the scala tympani were effective in preventing degeneration of the auditory nerve.

## MATERIALS AND METHODS

### Alginate Bead Production

Alginate beads were prepared using an ionic gelation technique. Two percent (w/v) sodium alginate (KelHV; ISP, Silverwater, NSW, Australia) with or without 1% (w/v) heparin (Sigma-Aldrich, Castle Hill, NSW, Australia) was dissolved in MilliQ water and mixed overnight until dissolved forming a gel-like solution. Subsequently, the gel was extruded through a 30-gauge needle into a bath of 0.1 M CaCl<sub>2</sub>, precipitating calcium alginate spheres. The beads were allowed to mature

with gentle stirring for 20 minutes and then washed three times over 30 to 40 minutes with 0.9% (w/v) NaCl. This method produced large beads in the order of 2 to 2.5 mm. For *in vivo* experiments, a stream of high-pressure CO<sub>2</sub> was passed through the needle as the gel was extruded, precipitating smaller (0.5–1 mm) alginate-heparin beads. The beads were produced using sterile solutions and equipment in a laminar flow hood and finally ultraviolet irradiated for 30 minutes. All beads were made fresh for *in vivo* experiments with a total dose of approximately 1.5 µg of NT-3 for each "loaded bead" experiment (3).

### In Vitro NT-3 Release Studies

Alginate and alginate-heparin beads were tested for loading and release of NT-3 *in vitro* using an enzyme-linked immunosorbent assay (ELISA). The aim was to produce the most effective loading and release profile for our delivery system. Beads with diameters of 2 to 2.5 mm were loaded with human recombinant NT-3 (Chemicon, Boronia, Vic, Australia) by incubating the beads overnight at 37°C in Ringer's solution containing NT-3. Uptake of NT-3 was quantified by measuring the differences between the initial and leftover loading solutions by a commercially available NT-3 ELISA kit (Promega, Annandale, NSW, Australia). After loading, the beads were then incubated in Ringer's solution at 37°C, and eluants were sampled at regular intervals for up to 5 days to examine the release profile of NT-3 from the beads.

Three experiments were performed varying the amount of NT-3 loaded and the number of alginate and alginate-heparin beads. Each experiment varied in the number of beads and the concentration of the load solution as detected in Table 1. All elution samples were tested in triplicate measures to reduce error. The percentage of release for each type of bead, in each experiment, was calculated by dividing the quantity released by the quantity of uptake. To increase the power of subsequent statistical analyses, the results of the three experiments were combined.

### In Vivo Experimental Design

The main measure of outcome was the density of SGNs surviving after 4 weeks of treatment (NT-3 loaded beads, unloaded beads, and untreated controls). SGNs degenerate, and the majority undergo apoptosis after the loss of hair cells sustained during an ototoxic injury. Successful NT-3 delivery was inferred from an increase in the SGN density in NT-treated ears and compared with untreated ears.

Seven-day deafened adult pigmented guinea pigs ranging in weight from 650 to 910 g were used for *in vivo* experiments. Two methods of delivery of the beads were tested, round window and intracochlear (Table 2). All contralateral cochleae were analyzed as untreated controls.

TABLE 1. *In vitro* NT-3 release studies

Experiment no.	Number of beads used in study	NT-3 loading concentration (µg/ml)	Loading volume (µl)	Uptake analysis	Release analysis: ELISA time intervals (days)
1	40 plain alginate	13.1	100	No	0.25,1,2,3,4,5
1	40 alginate-heparin	13.1	100	No	0.25,1,2,3,4,5
2	30 plain alginate	5.94	200	Yes	0.25,1,2,3,5
2	30 alginate-heparin	5.94	200	Yes	0.25,1,2,3,4,5
3a	10 alginate-heparin	30.5	100	Yes	0.25,1,2,3,4,5
3b	10 alginate-heparin	12.2	100	Yes	0.25,1,2,3,4,5

NT, neurotrophin; ELISA, enzyme linked immunosorbent assay.

TABLE 2. *In vivo* experiments

Bead placement	NT-3 loading	Guinea pigs
Round window	Loaded beads	5
	Unloaded beads	4
Intracochlear	Loaded beads	5
	Unloaded beads	4

NT, neurotrophin.

### Guinea-Pig Anesthesia

All surgical or auditory brainstem procedures were conducted under anesthesia using an intramuscular injection of ketamine (60 mg/kg) and xylazine (4 mg/kg). Anesthetic was reversed with antisedan at 0.02 to 0.2 ml/kg. For alginate bead implantation procedures, the animals were given a perioperative antibiotic, baytril, and an additional analgesic, rimadyl (4 mg/kg). All local skin infiltration was with 0.3 ml of 2% lignocaine hydrochloride. All procedures and animal care was performed with the approval and guidelines set by the Animal Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital.

### Guinea-Pig Auditory Brainstem Responses and Deafening

Hearing states of all pre- and postdeafened animals were estimated from click-evoked auditory brainstem responses (ABR) (38). Pigmented guinea pigs with a click-evoked ABR threshold of less than 48 dB peak-equivalent sound pressure level (normal hearing) were deafened by cannulation of the jugular vein or its large tributaries and delivery of furosemide (100 mg/kg) followed up by a subcutaneous injection of kanamycin sulphate (400 mg/kg) to produce a bilateral and symmetrical degeneration of the cochlear sensory epithelium (39). A postdeafening ABR was performed to confirm deafness, defined by a click-evoked ABR threshold of 93 dB peak equivalent sound pressure level or more. Animals not meeting this criterion were excluded from the study.

### Alginate Bead Implantation

Seven days after deafening, the guinea pigs were implanted with either small loaded (approximately 1.5 µg of NT-3 per bead) or small unloaded beads. The bulla was approached dorsally and the round window visualized under the bony ridge of the facial nerve. For round window implantation, the beads were placed adjacent to the round window (4–5 small beads) and secured with a muscle/fascial graft. The scala tympani was implanted by perforating the round window, passing 4 to 5 small beads into the basal turn of the cochlea, and sealing the round window with a muscle graft. Beads were implanted for 28 days.

### Cochlear Processing and Histology

Guinea pigs were killed with an overdose of sodium pentobarbitone and perfused by way of the intracardiac route with 0.9% (w/v) saline containing 0.1% (v/v) heparin sodium and 0.025% (w/v) sodium nitrite followed up by 10% (v/v) neutral buffered formalin. The cochleae were removed and postfixed in 10% (v/v) neutral buffered formalin for a further 24 hours. The cochleae were then decalcified in 4% (w/v) ethylenediaminetetraacetic acid. For SGN density measurements, cochleae were embedded in paraffin and sectioned at 5 µm around the round window and through the mid-modiolar plane. For histologic examination of cochleae around the

implantation site, cochleae were embedded in resin and sectioned serially at 125 µm. All sections were stained with hematoxylin-eosin.

### Data Analysis

All histologic sections were analyzed for inflammatory and fibrotic changes, especially in the vicinity of the implanted beads. SGN densities of individual cochlear turns were counted using National Institute of Health Scion Image analysis software. Slides were blinded and randomized before counting, and three mid-modiolar sections were counted and averaged for each cochlea. For each section, SGN densities were analyzed for each modiolar turn.

All the data were entered twice for consistency. Mann-Whitney and Kruskal-Wallis nonparametric analysis of variance were used to test for differences in the *in vitro* NT-3 release study. One-way analysis of variance was used to test for the differences between the three implantation groups (no treatment, round window beads, and intracochlear beads). Ninety-five percent confidence intervals are presented. Multiple comparison tests were also performed to identify which paired treatment was significant. Statistical analysis was performed using SPSS statistical software (V 8.0, Chicago, IL, U.S.A.).  $p < 0.05$  was considered to be statistically significant.

## RESULTS

NT-3 release from alginate beads was examined *in vitro* and measured using ELISA. Three *in vitro* experiments were performed to evaluate the loading and release properties of plain alginate and alginate-heparin beads (Table 1). NT-3 loading profiles were evaluated from Experiments 2 and 3. These experiments revealed a high uptake for plain alginate (98.0%) and alginate-heparin beads (98.8%) (Table 3).

The release profile is demonstrated in Figure 1. There was no difference in total amount of release of NT-3 from the plain alginate or heparin-alginate beads over a 5-day period ( $p = 0.95$ ). The NT-3 detected, expressed as a percentage of initial uptake, over the 5 days was 0.33% for plain alginate and 0.34% for alginate-heparin beads. However, there was a significant difference between the two beads in the release profile over the time period ( $p = 0.001$ ). Plain alginate beads released the majority of the NT-3 in the first 6 hours (94% of the total release over the 5 day period), whereas alginate-heparin beads released NT-3 more slowly over the 5 days (Fig. 1).

TABLE 3. NT-3 uptake analysis

Experiment no.	Number of beads used for study	NT-3 load concentration (µg/ml)	Postloaded	
			NT-3 concentration (µg/ml)	Percentage NT-3 uptake
2	35 plain alginate	5.94	0.119	98.0
2	35 alginate-heparin	5.94	0.043	99.3
3	20 alginate-heparin	30.5	0.494	98.4
3	20 alginate-heparin	12.2	0.161	98.7

NT, neurotrophin.

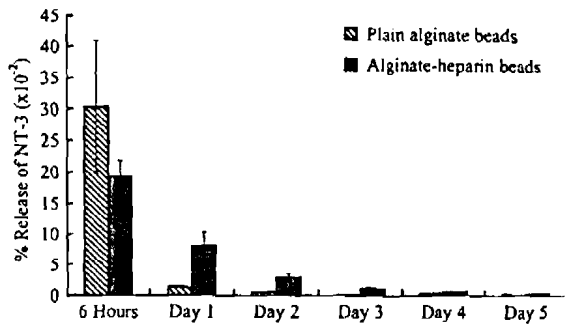


FIG. 1. Release of NT-3 from plain alginate or alginate-heparin beads. Plain alginate or alginate-heparin beads were loaded with NT-3 and placed in Ringer's solution, which was monitored for release of NT-3 by ELISA over 5 days. There was a significant difference ( $p = 0.001$ ) in the release profile between the two groups, with the alginate-heparin beads demonstrating a steadier and slower release than the plain alginate beads. Release is expressed as a percentage of NT-3 taken up by the beads during loading. Error bars represent standard error of the mean.

#### Histologic Analysis of Alginate Beads In Vivo

Macroscopic examination of the inner ear at 4 weeks showed that the beads had lost some of their morphologic shape and degraded into a gelatinous paste. The round window and basal cochlear turn were analyzed histologically for evidence of local tissue reaction to the beads. Hematoxylin-eosin stained sections revealed no significant tissue inflammation or fibrosis. Furthermore, no remnants of the beads were detected; however, this may have been a result of tissue processing.

#### In Vivo Results of NT-3 Loaded Alginate Beads

Spiral ganglion cells of all deafened animals, including those treated with NT-3, exhibited cells with a range of histopathologic states, from apparently normal to pyknotic cells, some of which lacked nuclei. The criterion for a therapeutic effect of the NT-3 was an increase in the density of spiral ganglion cells. Only neurons with a clearly defined nucleus were considered to be potentially viable and counted. Mid-modiolar spiral ganglion cell density measurements were made from both the round window and the intracochlear bead groups. The densities from each cochlear turn were measured in triplicate and subsequently averaged. Mid-modiolar SGN density measurements were averaged across all the mid-modiolar turns of the cochlea, and comparisons were made for each of the individual experimental groups (Table 4).

In both the round window and intracochlear implantation sites, cochleae treated with alginate beads loaded with NT-3 had greater SGN survival than untreated or cochleae implanted with unloaded beads (Fig. 2). There was no significant difference in SGN densities between the unloaded and untreated contralateral ears.

In the round window group, a significantly higher SGN density difference was observed in the NT-3 loaded beads ( $p < 0.001$ , mean = 1,060, SD = 120) compared

TABLE 4. Combined mid-modiolar SGN density results

	Round window		Intracochlear		Combined	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
Untreated	—	—	—	—	770	(728, 812)
Unloaded beads	686	(603, 770)	702	(585, 821)	695	(628, 762)
Loaded beads	1,060	(1,009, 1,112)	984	(899, 1,069)	1023	(975, 1,071)

CI, confidence interval; SGN.

with the unloaded beads (mean = 690, SD = 180) and untreated cochleas (mean = 770, SD = 190). A significantly higher SGN density difference was also observed in the intracochlear NT-3 loaded bead group (mean = 980, SD = 210) compared with the unloaded beads ( $p < 0.001$ , mean = 700, SD = 250) and untreated cochleas (mean = 770, SD = 190) (Fig. 2).

#### Effect of Beads on Individual Cochlear Turns

Individual cochlear turn auditory nerve density comparisons were made for round window and intracochlear bead implantations. For both methods of bead delivery, there was significantly higher SGN protection in the treated ear through all the cochlear turns ( $p < 0.001$ ); however, there was no difference noted between the turns (Fig. 3). Although in the intracochlear test group there was a trend toward greater protection in the lower turns, this was not significant.

## DISCUSSION

This investigation established alginate beads to be an effective delivery vehicle for NT-3. The in vitro studies demonstrated that alginate encapsulates a large percentage

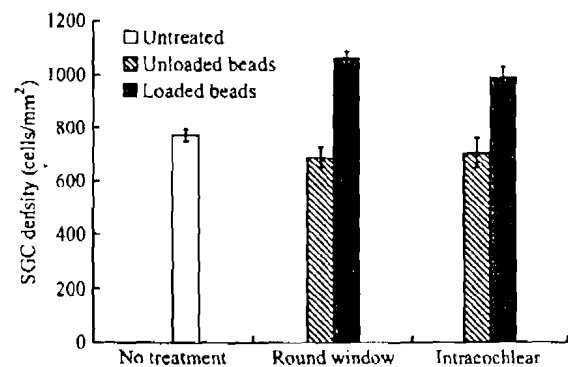


FIG. 2. Comparison of round window and intracochlear methods of bead implantation. Alginate beads loaded either with a control solution (unloaded beads) or with NT-3 (loaded beads) were implanted either onto the round window membrane of deafened guinea pigs or within the basal turn of the cochlea (intracochlear). NT-3 loaded beads implanted at both sites were effective in maintaining the survival of SGNs compared with unloaded beads or cochleae that received no treatment. Error bars represent standard error of the mean.

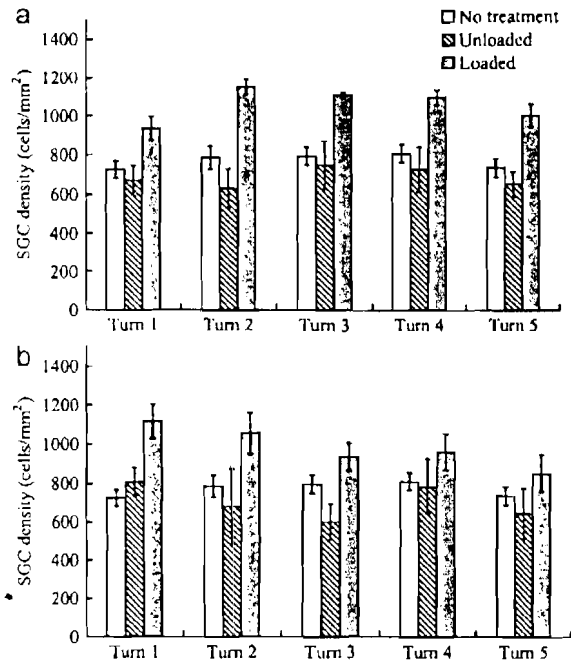


FIG. 3. SGN density in each turn of the cochlea after round window and intracochlear alginate bead implantation. NT-3 loaded or unloaded beads were implanted at the round window (A) or within the basal turn of the cochlea (B) of deafened guinea pigs for 28 days. SGN density was measured at each turn of the cochlea. NT-3 loaded beads demonstrated greater survival of SGNs through all the cochlear turns compared with unloaded beads or untreated cochleae. For the intracochlear implantations, this effect was most prominent in the basal turns of the cochlea. Error bars represent standard error of the mean.

of NT-3, in the vicinity of 98% to 99%, and the addition of heparin favorably altered the release kinetics to a slower and steadier release over 5 days (Fig. 1). This may potentially reduce the toxicity that could be associated with a large initial NT release from alginate.

The amount of NT-3 released in vitro (0.33% of initial uptake) was significantly less compared with its uptake. One possible explanation is that the beads have not yet fully degraded by the end of the release study and that NT-3 is still present within the bead. If so, the potential NT-3 deliverable by the bead exceeds that shown from our release studies, and delivery may depend on the rate of degradation in different environments. Another possible explanation is that NT-3 may be bound to alginate breakdown products and not freely available for ELISA measurements. This is suspected because when the beads were degraded in a calcium-chelating environment of ethylenediaminetetraacetic acid to quantify the initial uptake of NT-3, the measured amount was 0.33%, considerably less than the 96% anticipated.

The in vivo work has demonstrated the alginate-heparin beads to be an effective delivery vehicle for NT-3, with a significant protective effect upon the auditory nerve. The protective effect was measured as an increase in the density of surviving spiral ganglion cells, although

the protection was not complete because treated animals had poorer spiral ganglion cell survival than normal controls.

Other protective effects that may be achieved by administration of NTs include an increase in soma size (40) and preservation or resprouting of peripheral dendrites (41). Functional effects of NTs include a reduction in the threshold of electrically evoked auditory brainstem potentials (42,43). Therefore, there may be benefits in delivering NTs to cochlear implant recipients, both in terms of neuronal survival and function. The current study provides a model for the development of a clinically applicable delivery method.

Although in this study the alginate beads were used in deafened animals, it is anticipated that they may have a role in drug delivery to the hearing ear. Alginate could potentially impact upon hearing by exerting toxic effects upon the inner ear or alternatively by causing a conductive hearing loss when an alginate bead is placed upon the round window. The latter should be temporary, and resolve when the bead degrades. Further investigation is required to explore the effects of alginate upon hearing.

This investigation has demonstrated several potentially important advantages of the delivery vehicle. First, alginate-heparin beads are nontoxic to neurons because there was no significant difference in SGN densities between cochleae implanted with unloaded beads and the untreated ears (Figs. 2 and 3). Second, the beads are biodegradable. They were found to degraded into a gelatinous semiformed paste after 4 weeks in vivo. Third, the NT-3 loaded beads are efficacious in promoting neural survival when applied either to the round window or the basal turn of the cochlea (Figs. 2 and 3). This protection was universal for all the turns of the cochlea (Fig. 3). Although the protection from the ototoxic insult was not complete, with further refinements of the delivery system, we may be able to provide a greater degree of release and protection. Such neuronal protection demonstrated by NT-3 loaded alginate-heparin beads could be applied to other areas of nerve regeneration research such as in the spinal cord and peripheral nerves (44).

Finally, this preliminary study has demonstrated that round window and intra-cochlear application of alginate-heparin beads are equally efficacious in delivering NT-3 to the cochlea (Figs. 2 and 3). The round window route may have potential clinical applications in delivering drugs, NTs, and viral vectors in a simple outpatient setting under local anesthesia. Small beads could be manufactured, loaded with drugs, and delivered to the round window by way of a tympanotomy performed under local anesthesia. Although not directly applicable in clinical settings, the intracochlear route demonstrated that NT-3 release from a polymer-coated cochlear implant electrode is feasible. This opens up the potential for the delivery of drugs that are impermeable to the round window or even stem cell delivery to the cochlea. The current study is the first to demonstrate efficacious NT delivery bound to a polymer placed adjacent to, or within, the inner ear.

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**Author/s:**

Noushi, Fanoosh;Richardson, Rachael T.;Hardman, Jennifer;Clark, Graeme M.;O'LEARY, STEPHEN

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